RNAi-based Therapeutic Strategies for Metabolic and Inflammatory Diseases

Dr. Michael Czech

Dr. Michael Czech is a Professor and Chair of Molecular Medicine and a Professor of Biochemistry and Molecular Pharmacology at the University of Massachusetts Medical School in Worcester, Massachusetts. He received his Ph.D. in Biochemistry from Brown University. Dr. Czech has published nearly 300 papers and has served, or is presently serving, on the editorial boards of dozens of journals. He has also served on the NIH endocrinology study section and on the Howard Hughes Medical Institute review panel. Dr. Czech was the recipient of the Grodsky Basic Research Award from the Juvenile Diabetes Research Foundation International in 1997, the 1998 Elliot P. Joslin Medal in diabetes research, and the 2000 Banting Medal of the American Diabetes Association. The following are highlights from the scientific presentation that Dr. Czech gave to the NIDDK's Advisory Council in May 2008.

Understanding the process by which the body slowly becomes resistant to the hormone insulin, as is the case in type 2 diabetes, is critical to developing effective therapeutics for the disease. Recent research has revealed a link between insulin resistance and the inflammatory response of the immune system. As the body takes in excess calories, fat cells, known as adipocytes, increase in size to store the extra fat. Eventually, the adipocytes become overloaded and begin to release molecules that attract inflammatory cells, specifically macrophages. Macrophages are important in initiating the inflammatory response; they engulf foreign pathogens, such as bacteria or yeast, and secrete molecules that affect the behavior of other immune system cells and that attract additional inflammatory cells to the site of the pathogen. However, inflammation does not only occur when there is an obvious infection. A chronic state of inflammation can occur when macrophages are continually recruited to adipocytes, as in the case of obesity. In this state and with the adipocyte's ability to store fat exceeded, the muscle begins to take up the excess fat. The build up of fat in the muscle disrupts the ability of insulin to stimulate the transport of glucose (sugar) from the blood into muscle, leading to insulin resistance.

Dr. Czech's presentation moved from an initial fundamental discovery to an innovative strategy for its clinical application. He discussed his approach to understanding how cells become resistant to insulin and the role of the inflammatory response in insulin resistance. He shared how his laboratory has utilized a revolutionary technique—ribonucleic acid (RNA) interference, or RNAi—to identify novel molecules critical to these processes. Dr. Czech and his team are exploring the use of this technique as a potential therapy for insulin resistance. Dr. Czech also remarked that this research was made possible by NIDDK's Diabetes Genome Anatomy Project (DGAP), a unique and multi-dimensional initiative for basic research in diabetes. DGAP was designed to facilitate interactions and coordinate a number of investigators at multiple institutions, with projects aimed at understanding the interface between insulin action, insulin resistance, and the genetics of type 2 diabetes.

Using RNAi To Identify Novel Proteins in Insulin Resistance

Dr. Czech and his colleagues sought to identify proteins that mediate the interactions between

adipocytes and macrophages and to understand their role in the balance of blood glucose and fat levels. Such information could reveal new drug targets to break the link between obesity and insulin resistance. To uncover these proteins, the scientists used a technique based on the phenomenon of RNA interference. This technique involves designing small molecules, known as small interfering RNAs or siRNAs, that reduce levels of a specific protein by interacting—or interfering—with the genetic material that encodes the protein, to prevent the protein from being made. The scientists thus could design specific siRNAs to reduce the level of a protein and see whether insulin-mediated glucose uptake was affected. With this technique, they screened hundreds of different proteins in mouse adipocytes to determine whether any had a role in insulin action.

Several specific proteins were identified that Dr. Czech and his colleagues never expected to be involved in insulin resistance. One of these is called MAP4K4 (shorthand for mitogen activated protein 4 kinase). Dr. Czech's laboratory subsequently demonstrated that MAP4K4 blocks insulin-stimulated glucose transport through a mechanism that also involves an inflammatory response protein called TNF-alpha. This positions MAP4K4 at the interface between adipocytes, where MAP4K4 can be found, and macrophages, which secrete TNF-alpha. MAP4K4 is also located in other types of cells, and Dr. Czech's laboratory and others have identified additional roles for this protein in muscle and in macrophages, placing MAP4K4 in three key tissues involved in insulin resistance in obesity.

Developing RNAi as a Potential Therapeutic

Once a role had been identified for MAP4K4 in inflammation and insulin-dependent glucose uptake, Dr. Czech wanted to explore whether targeting this protein, using the power of RNAi, could have therapeutic potential for diabetes. The investigators decided to target levels of MAP4K4 protein in macrophages, rather than in muscle or adipocytes,

because they hypothesized that insulin resistance results from the stimulation of inflammation by MAP4K4 in macrophages. In addition, because macrophages—and inflammation—are involved in many diseases, such as rheumatoid arthritis, colitis, inflammatory bowel disease, cardiovascular disease, and atherosclerosis, developing a strategy for therapy in the macrophage might be applied to many other diseases.

Dr. Czech explained that RNA interference as a potential therapeutic may have several advantages over traditional small molecule drugs, which interact with proteins. In traditional drug development there are a relatively limited number of proteins that can be targeted, as the small molecules normally tested as drug candidates are only effective if they can bind (attach) to the targeted protein. By contrast, RNAi works by interfering with genetic material encoding proteins, not the proteins themselves, and scientists think that there may be fewer structural constraints for this type of interaction. With RNAi, therefore, levels of any protein encoded in the human genome could theoretically be targeted and reduced. Second, traditional small molecule drugs can sometimes bind non-targeted proteins. Because siRNAs are extremely specific in their targets, off-target—and potentially toxic—effects can be minimized. Additionally, siRNAs are made from materials that are native to the body and have not shown toxicity thus far in animal models.

As Dr. Czech noted, an ideal therapeutic would be delivered orally for the patient's ease. An orally delivered drug faces many obstacles on its way to the target tissue: it needs to pass through the acidic environment of the stomach, be absorbed by the gut, and enter the bloodstream. An ideal therapeutic would be specifically delivered to the targeted tissues, thereby avoiding any toxicity due to misdelivery. To address these challenges, Dr. Czech took advantage of special cells called "M cells," which are located within the small intestine, and devised a way to get siRNA to these cells.

The M cells constantly sample the digestive cavity of the intestine looking for particles like bacteria and yeast that may have been ingested. Upon finding these, M cells are able to bind the particles, internalize them, and expel them where nearby macrophages are waiting to devour them. With this system, Dr. Czech utilized the normal biology of the intestine to efficiently direct his RNAi therapeutic to the macrophages.

Dr. Czech's laboratory needed to generate a safe vehicle to deliver the siRNA to an animal being studied. Their efforts led to the development of hollow, porous, tiny (micron-sized) shells made of a substance called beta1,3-D-glucan, which is recognized by proteins on both the M cells and the macrophages, permitting these cells to take in the shells. Beta1,3-D-glucan is a non-toxic material made by yeast cells and has been sold as a human dietary supplement for many years. Layering the siRNA within the hollow center of the shell allows five to six layers of siRNA to be put into each of these particles. Therefore, the scientists could use multiple combinations of siRNA at one time and target several different genes, or use one siRNA at a higher dose. Dr. Czech and his colleagues termed these shell particles "GeRPs" or Glucan-encapsulated siRNA particles.

Proof of Principle: Using RNAi To Target MAP4K4 in Animals

This technology required multiple tests to determine whether it could be used as a potential therapeutic in animals. To begin, Dr. Czech and his colleagues needed to ascertain whether the macrophages would even ingest the GeRPs— the first step in this strategy. To do this, the scientists added a fluorescent label to the GeRPs and gave them orally to mice. Using a fluorescence microscope, they were able to see that the macrophages had taken in the GeRPs and that a single macrophage could ingest multiple GeRPs. Another exciting aspect of this technology is that it harnesses the macrophages in the gut to transport the GeRPs. These macrophages are part of the body's lymphatic system, which enables them to travel throughout the body. This prompted Dr. Czech and his colleagues to assess if they could find GeRPs inside macrophages located in various tissues of the mouse body. After 8 days of feeding the mice GeRPs, the scientists observed the fluorescent GeRPs in the lungs, liver, and spleen. From this result, Dr. Czech and his laboratory concluded that they are able to target multiple tissues in the mouse body with this technology.

Dr. Czech's next step was to examine whether GeRPs with siRNA directed to MAP4K4 led to a reduction in the levels of MAP4K4 proteins within the tissues of the mice. In spleen, liver, and lung, the scientists were able to see a reduction in the levels of MAP4K4 as they had hoped. Did this reduction in MAP4K4 protein levels affect the inflammatory response though, as Dr. Czech had predicted? The scientists again fed the mice GeRPs with siRNAs to MAP4K4 and then gave the animals a toxic chemical that mimics a bacterial infection in order to stimulate the inflammatory response. When mice without the siRNAs were given this chemical, their macrophages stimulated an excessive inflammatory response, leading to a very large release of the inflammatory protein TNF-alpha, which was fatal to the animals. However, by feeding the mice siRNA to MAP4K4, Dr. Czech and his colleagues were able to block this storm of TNF-alpha, halting the inflammatory response to the chemical, and protecting the mice. This exciting result demonstrated that the orally administered siRNAs were not only delivered to the correct cell, the macrophage, and carried to multiple tissues, but that these siRNAs also targeted MAP4K4 specifically and altered the mouse inflammatory response.

Does using this technology to target MAP4K4 reduce inflammation in fat tissue and affect insulin-mediated glucose transport into cells? For these preliminary experiments, Dr. Czech and colleagues used obese mice that are highly insulin-resistant and delivered MAP4K4 siRNA-containing GeRPs to the mice by

injection. The scientists looked at various tissues to determine the location of GeRP-filled macrophages and evaluated whether the mice were still resistant to insulin with a test called a "glucose tolerance test." They found, to their surprise, that the fat tissue of these mice was the main tissue that had macrophages with GeRPs in them. This indicated that, in these obese mice, the primary inflamed tissue is the fat tissue— macrophages are largely recruited to this tissue. Dr. Czech and his laboratory also observed a decrease in levels of MAP4K4 protein in the macrophages recovered in this tissue. In addition, these mice were better able to metabolize glucose, indicating that the insulin resistance of these obese mice could be ameliorated. These experiments suggested that delivery of MAP4K4 siRNA to obese mice could have a profound effect on glucose metabolism throughout the body.

Conclusion

Dr. Czech's presentation illustrated the power of RNAi technology to identify novel proteins involved in insulin resistance. These proteins are potential targets for drug therapy, as they are found at the interface between fat cells, muscle, and the inflammatory response. One particular protein, MAP4K4, is especially interesting due to its location in all of these tissues. In addition, Dr. Czech showed his laboratory's approach to using siRNAs as a therapeutic modality. By targeting siRNA to MAP4K4 within the macrophages of a mouse with an innovative delivery vehicle, the scientists were able to both block the inflammatory response and alter the insulin resistance in obese mice. Thus, Dr. Czech and his colleagues have developed a technology to deliver RNAi *in vivo* in mice. They plan to build on the studies to determine whether the therapeutic has a similar result in other animals. Dr. Czech's research reveals the exciting potential for a new method of therapy for numerous diseases, including type 2 diabetes.